A PROSPECTIVE ROLE OF SPECIFIC FOCAL ADHESION KINASE PHOSPHORYLATION SITES AS PROGNOSTIC MARKERS IN CANCERS OF BREAST, COLON, LIVER AND LEUKAEMIA

By

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In the name of Allah

The most beneficent the most merciful

THIS THESIS IS DEDICATED

TO

MY FATHER AND MOTHER FOR DOING THEIR BEST

TO EDUCATE ME

MY MARTYRED COLLEAGUES AND GAZA

CHILDREN WHO LOST THEIR LIFE IN

THE ZIONIST ATTACKS

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LIST OF ABBREVIATIONS

AML	Acute myeloid leukaemia
BSA	Bovine serum albumin
CCD-18Co	Normal colon cell line
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ES	Oestrogen receptor
FAC	Focal adhesion complex
FAK	Focal Adhesion Kinase
FAK-CD	Focal Adhesion Kinase C-terminal domain
FAT	Focal adhesion targeting
FBS	Foetal Bovine Serum
FRNK	FAK-related-non-kinase domain
HCT 116	Colorectal carcinoma cell line
Hep G2	Hepatocellular carcinoma cell line
HGF	Human growth factor
HT-29	Colorectal adenocarcinoma cell line

MAPK	Mitogen-activated protein kinase
MCF 10A	Non– tumourigenic breast epithelial cell line
MCF7	Oestrogen positive breast adenocarcinoma cell line
MDA-MB-231	Oestrogen negative breast adenocarcinoma cell line
MMPs	Matrix metalloproteinases
MT1-MMP	Membrane type 1- matrix metalloproteinases
NRPTKs	Non receptor protein tyrosine kinase
p130Cas	Crk-associated substrate
P13-kinase	Phosphatidylinositol 3-Kinase
PBS	Phosphate buffer saline
PBS-MLK	Phosphate buffer saline-Milk
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PR	Progesterone receptor
PTKs	Protein tyrosine kinases
RIP	Receptor interacting protein
RPTKs	Receptor protein tyrosine kinase
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser 732	Serine 732
Ser 843	Serine 843
Ser 910	Tyrosine 910
T-47D	Oestrogen positive breast ductalcarcinoma cell line
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
Tyr 397	Tyrosine 397
Tyr 407	Tyrosine 407
Tyr 576	Tyrosine 576
Tyr 577	Tyrosine 577
Tyr 861	Tyrosine 861
Tyr 925	Tyrosine 925
U-937	Histiocytic lymphoma cell line
VEGFR-3	Vascular endothelial growth factor receptor-3

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Suatu Perspektif Fungsi Spesifik Tapak Pemfosforilan *Focal Adhesion Kinase* Sebagai Penanda Prognostik dalam Kanser Payu Dara, Kolon, Hati dan Leukemia

ABSTRAK

Focal Adhesion Kinase (FAK) merupakan suatu protein kinase tirosin bukan reseptor bersaiz 125 kDa. FAK memainkan peranan penting dalam pengawalaturan pelbagai aktiviti sel seperti migrasi, perekatan, proliferasi dan pertumbuhan. Dilaporkan bahawa FAK mempunyai dua fungsi, iaitu sebagai protein kerangka dan protein kinase. Analisis pada paras protein dan sel menunjukkan ekspresi FAK yang berlebihan dan pemfosforilan FAK pada residu khusus dalam kebanyakan tumor. Walau bagaimanapun, kebanyakan data ini masih kurang jelas dan memerlukan kajian lanjut. Dalam kajian ini, kami memeriksa total FAK dan pemfosforilan FAK pada pelbagai residu dalam sel kanser payu dara, kolon, hati dan leukemia. Di samping itu, kami juga membandingkannya dengan sel normal menggunakan analisis pemblotan Western dan analisis imunopendarflour. Kami mencadangkan bahawa tahap fosforilasi FAK yang tinggi pada Tyr 397 dan Tyr 861 sangat diperlukan dalam dalam sel kanser payu dara yang lebih invasif. Kami juga mendapati bahawa MDA-MB-231, sel kanser payu dara yang tidak mengekspresikan reseptor estrogen, menunjukkan fosforilasi FAK yang tinggi pada Tyr 397 dan Tyr 861. Ekspresi fosfo-FAK (Ser 910) yang tinggi dikesan dalam sel kanser payu dara yang kurang invasif (T-47D dan MCF-7) dibandingkan dengan MDA-MB-231 yang lebih invasif. Berkenaan dengan kanser kolon, kami mendapati sel kolon yang mengalami pembezaan yang sederhana dan kurang invasif, HT-29, menunjukkan tahap FAK yang lebih rendah dibandingkan dengan sel kanser kolon yang tidak membeza dan

lebih invasif, HCT 116. Fosfo-FAK (Tyr 397) diekspresi dengan lebih tinggi dalam HT-29 berbanding HCT 116. Fosforilasi FAK pada Ser 910 dikesan dalam sel normal dan sel yang mengalami pembezaan, CCD-18Co dan HT-29 masing-masing, tetapi tidak dikesan dalam sel yang lebih agresif, HCT 116. Penemuan ini mencadangkan bahawa fosforilasi FAK pada dua residu tirosin (Tyr 397 dan Tyr 861) mungkin suatu keperluan penting dalam memberikan ciri invasif kanser memandangkan aras yang tinggi diperhatikan dalam titisan sel yang lebih invasif. Berbeza dengan kanser payu dara, protein FAK mungkin diperlukan terutamanya dalam regulasi sel kolon normal dan sel kolon yang terbeza, seperti CCD-18Co dan HT-29 masing-masing. Fosforilasi FAK pada Ser 910 mungkin mempunyai kesan perencatan dalam kanser payu dara, memandangkan sel yang kurang invasif, T-47D menunjukkan aras fosforilasi yang lebih tinggi pada residu ini. Kedua-dua Tyr 397 dan Tyr 861 juga tidak terfosforilasi. Berdasarkan kajian kami, dicadangkan bahawa fosforilasi FAK pada Ser 910 mungkin memainkan peranan yang penting dalam mengekalkan sebahagian daripada sifat-sifat yang berkait dengan fenotaip normal dan fenotaip yang berbeza.

A Prospective Role of Specific Focal Adhesion Kinase Phosphorylation Sites as Prognostic Markers in Cancers of Breast, Colon, Liver and Leukaemia

ABSTRACT

Focal Adhesion Kinase (FAK) is a 125 kDa non-receptor protein tyrosine kinase. FAK plays a pivotal role in the regulation of various cellular activities such as migration, adhesion, proliferation and growth. It has been reported that FAK has dual function as a scaffold and kinase protein. Analyses at the protein and cellular levels have demonstrated FAK overexpression and phosphorylation at specific residues in many tumours; however the data were mostly inconclusive and need further investigation. In this study, we set out to examine total FAK and phosphorylated FAK at various residues in breast, colon, liver cancer and leukemic cell lines in comparison to normal cell lines using Western blotting and immunofluorescent analyses. We propose that higher levels of phosphorylated FAK at Tyr 397 and Tyr 861 are highly required in the more invasive breast cancer. We also found that MDA-MB-231, breast cancer cells negatively expressing oestrogen receptor, exhibited higher FAK phosphorylation at Tyr 397 and Tyr 861. Higher expression of phospho-FAK Ser 910 was detected in the less invasive breast cancer cell lines, T-47D and MCF7, compared to the highly invasive MDA-MB-231. In regard to colon cancer, we found that moderately differentiated and less invasive colon cancer cell line, HT-29, expressed lower FAK level compared to the un-differentiated and more invasive colon cancer cell line, HCT 116. Phospho-FAK Tyr 397 was more expressed in HT-29 than in HCT 116. However, phosphorylation at Ser 910 was detected in normal and more differentiated cells, CCD-18Co and HT-29 respectively, but was undetectable in the more aggressive cells, HCT 116. These findings suggest that

FAK phosphorylation at the two tyrosine residues, Tyr 397 and Tyr 861, may indicate a crucial requirement of these sites of phosphorylation in breast cancer invasiveness, since higher levels were observed in the more invasive cell lines. Contrary to breast cancer, FAK protein may be mainly required in the regulation of normal and differentiation colon cells, such as CCD-18Co and HT-29 respectively. Phosphorylation of FAK at Ser 910 appeared to have an inhibitory effect in breast cancer, since the less invasive cells, T-47D showed a higher level of phosphorylation at this residue whereas, both Tyr 397 and Tyr 861, were not phosphorylated. Based on our study, it is suggested that FAK phosphorylation at Ser 910 may play an important in retaining some of the characteristics of normal and differentiated phenotypes.

CHAPTER ONE

INTRODUCTION

1.1 Cancer

Cancer is a genetic disease happens as a result of several mutations that happen due to abnormalities in DNA sequence (Futreal *et al.*, 2001). Usually, a mutation is followed by an accelerated cellular proliferation resulting in increasing mass of cells termed as tumour (Vogelstein & Kinzler, 1993). In the development of cancer, a cell undergoes a multistep process of events leading to producing a malignant cell. These events are involved in the suppression of tumour suppressor genes, activation of oncogens and DNA alterations (Choy *et al.*, 2004). One of the leading causes of cancer deaths is metastasis. Tumour metastasis is the spread of tumours to local and distant places away from the initiating tumour. It has been shown that tumour cell obtained from metastatic tumour displayed increased capacities of migration and invasion (Jin & Varner, 2004).

Cancer is mostly classified according to its organ, or cell type in which it starts. Also, it can be classified into wider categories, while the main categories are:

- Carcinoma - cancer of epithelial cells which initiates in tissues or skin that line or cover body organs.
- Sarcoma - cancer of the connective tissues, like cancer that begins in blood, muscle, bone, cartilage, fat, or other supportive or connective tissues.
- Leukaemia - cancer that produces a large number of abnormal blood cells in blood generating tissue, such as bone marrow.

- Lymphoma and myeloma - cancers that develop from cells originated from immune system.
- Central nervous system cancers - cancers that occur in tissues of central nervous system, such as brain and spinal cord (Montella *et al.*, 2001; Reya *et al.*, 2001; Bhowmick *et al.*, 2004).

It is postulated that cancer incidence is highly affected by the number of proliferating cells. Basically, cell proliferation depends on the number of dividing cells in addition to the rate of cell division in certain tissue (Albanes & Winick, 1988).

1.2 Tyrosine Kinases and cell signalling

Protein tyrosine kinases (PTKs) exhibit an important role in cancer signalling. PTKs transfer α-phosphate groups from ATP to tyrosine sites on signal transduction molecules in a process called phosphorylation. Usually, phosphorylation leads to switching various signalling pathways, and consequently stimulating cell proliferation, increasing cell migration capacity and prolonging survival. In addition, phosphorylation of tyrosine residues on signal transduction molecules serves to further activate PTKs and create binding sites for other cytoplasmic signalling molecules, such as phospho-tyrosine proteins and Src homology molecules (Olayioye *et al.*, 2000; Demetri *et al.*, 2002).

Receptor and non receptor protein tyrosine kinase families (RPTK and NRPTK respectively) are the main classes of PTKs. NRPTKs are basic components of signalling cascades activated by RPTKs and by other cell surface receptors. NRPTKs

are deficient in receptor-like features like an extracellular ligand-binding domain and transmembrane-spanning region, and normally they are localized in the cell cytoplasm (Hubbard & Till, 2000; Vlahovic & Crawford, 2003; Tsygankov, 2003). NRPTK family include Focal adhesion kinase (FAK), Src, Janus kinases (Jaks), Abelson tyrosine kinase (Abl), acetate kinase (Ack), C-terminal Src kinase (Csk), fps/fes related kinase (Fer/Fes), fyn-related kinase (Frk), Spleen tyrosine kinase (Syk) and Tec. (Gu & Gu, 2003). Figure 1.1 shows the involvement of RPTKs and NRPTKs in signals transmission. Following growth factors binding to their receptors, NRPTKs dimerization induces their autophosphorylation and conveys messages downstream to cytoplasmic and nuclear targets. Therefore, NRPTKs work downstream to RPTKs as they integrate RPTKs to transmit signals from extracellular matrix (ECM) to cell cytoplasm.

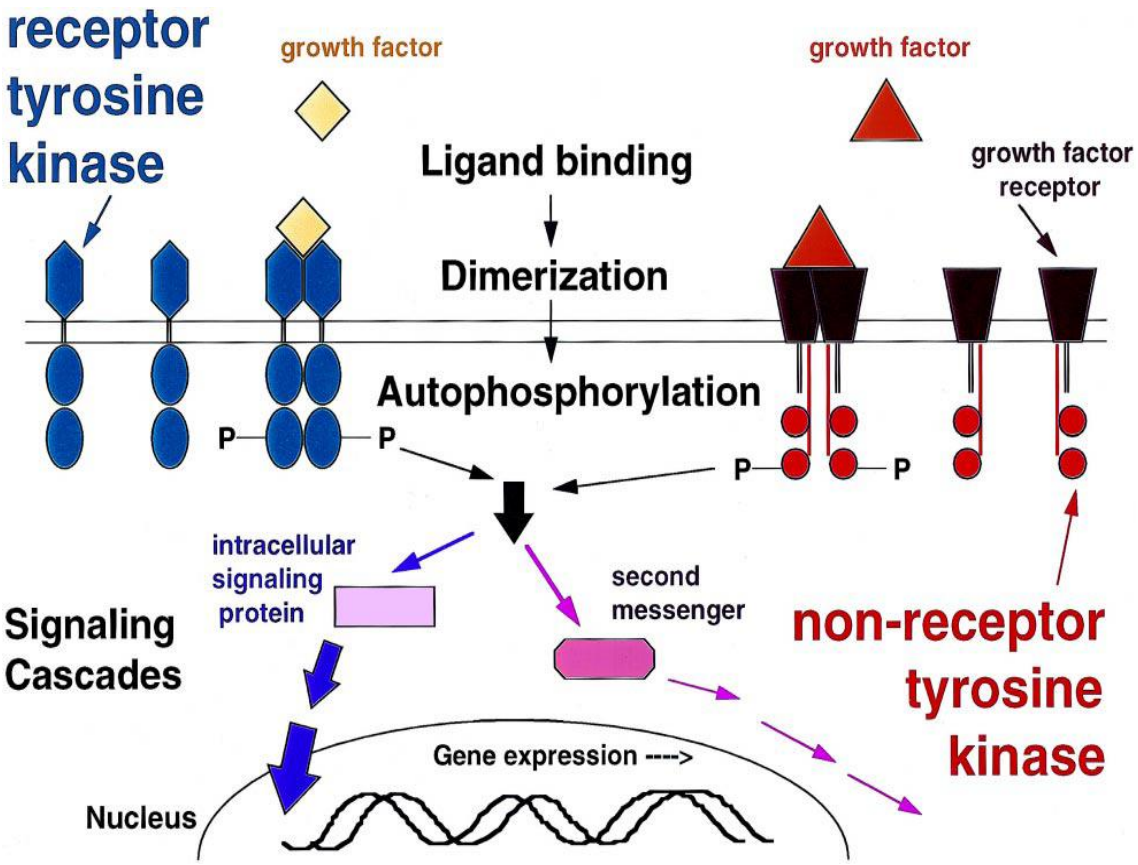

Figure 1.1: Signalling through tyrosine kinases (Adapted from Kolibaba and Druker, 1997).

1.3 Focal Adhesion Kinase

1.3.1 Focal Adhesion Kinase Discovery

Focal adhesion kinase (FAK) is a 125 KDa non-receptor cytoplasmic protein tyrosine kinase. FAK is localized to focal adhesion plaques where cellular interactions with extracellular matrix proteins take place. It was implied that FAK exhibits a crucial role in modulating several cellular signalling pathways (Schaller, 1996; Beviglia *et al.*, 2003). Two research groups headed by Steve Hanks, Jun-Lin Guan and Michael Schaller discovered FAK independently in 1992.

Prior to FAK discovery, previous researches were conducted to examine the involvement of Src in cancer signalling. FAK was identified as a substrate of the viral Src oncogen, and was known to localize at cell adhesion contacts where integrins assemble (Mitra *et al*,. 2005). It was shown that many human tumours expressed activated Src, and relevant FAK activation was related to specific human diseases. FAK activation was proposed to happen exclusively as a result of cellular transformations. Following activation, FAK was thought to promote tyrosine activation of substrates such as paxillin, and amplify mitogen activated protein kinase (MAP kinase) signalling in transformed cells. However, FAK inhibition was unable to restore transformed cells original phenotypes (Schaller, 2001; McLean *et al.*, 2005).

1.3.2 FAK Structure

Figure 1.2 illustrates FAK linear structure. FAK is encoded by PTK2 genes in humans (Gabarra-Niecko *et al.,* 2003). The encoded protein consists of three main domains, a non catalytic N-terminal domain, a central catalytic domain and a C-terminal domain. C-terminal domain consists of the focal adhesion targeting (FAT) sequence (Mon *et al.,* 2006). There are also docking sites for proteins with SH3 domain, located between catalytic domain and FAT sequence (Parsons, 2003). The N-terminal domain of FAK shows strong homology with structurally conserved FERM domains, which are present in various families of structural proteins like talin and protein 4.1, ezrin-radixin-moesin (FERM), as well as in JAK family kinases (Sun *et al.,* 2002). FERM domains were thought to mediate protein-protein interactions. Some binding partners of FAK-FERM domain have been reported, among others are β1 integrin and growth factor receptors (EGFR and PDGFR) (Sieg *et al.,* 2000). The N-terminal domain was thought to direct FAK interaction to signalling proteins and integrins clusters to regulate its catalytic activity (Parsons *et al.,* 2003). FERM domain-mediated interactions were also thought to have a role in the regulation of cell motility (Streblow *et al.,* 2003).

FAK tyrosine phosphorylation with subsequent activation was increased when FAK N-terminal domain was truncated. N-terminal domain truncation is followed by assembly of Src family of protein tyrosine kinases (PTKs), and binding of Src-SH2 domains to FAK at Tyr 397. FAK N-terminal domain truncation was thought to initiate autophosphorylation at FAK Tyr 397 leading to successive FAK phosphorylation at other tyrosine residues. These events were thought to fully

activate FAK molecule (Lietha *et al.,* 2007). Additionally, mutant cells with truncated N-terminal domain exhibited an increased level of cell migration (Schaller, 2001).

Previous report suggested a role of FAK N-terminal (FERM) domain in the suppression of p53 transcription, thus the FERM domain could have an anti-apoptotic activity leading to increase cell survival (Mitra and Schlaepfer, 2006). On the other hand, the N- terminal domain was shown to induce cell adhesion loss followed by apoptosis. Figure 1.3 shows the possible role of FAK N-terminal domain in the induction of apoptosis through adhesion loss. The FERM domain appeared to be involved in FAK nucleocytoplasmic translocation, which may lead to cellular rounding, detachment and then proceeding in apoptosis.

The FAK C-terminal domain has many proteins interaction sites through which FAK can be localized and directed to adhesion complex regions. Focal adhesion targeting domain (FAT) is a part of the FAK C-terminal region which binds to paxillin and talin proteins, and contributes to supporting FAK localization to focal adhesions (Martin *et al.,* 2002). FAK-related-non-kinase domain (FRNK) an autonomously expressed C-terminal domain of FAK was thought to inhibit FAK kinase activity contributing to the inhibition of cell spreading as well as chemotactic and haptotactic migration (Hildebrand *et al.,* 1993; Hildebrand *et al.,* 1995). Besides that, it was implied that FRNK suppresses growth-factor-stimulated signals transduction to MAP kinase (Hauck *et al.,* 2001; Taylor *et al.,* 2001; Parson, 2003).

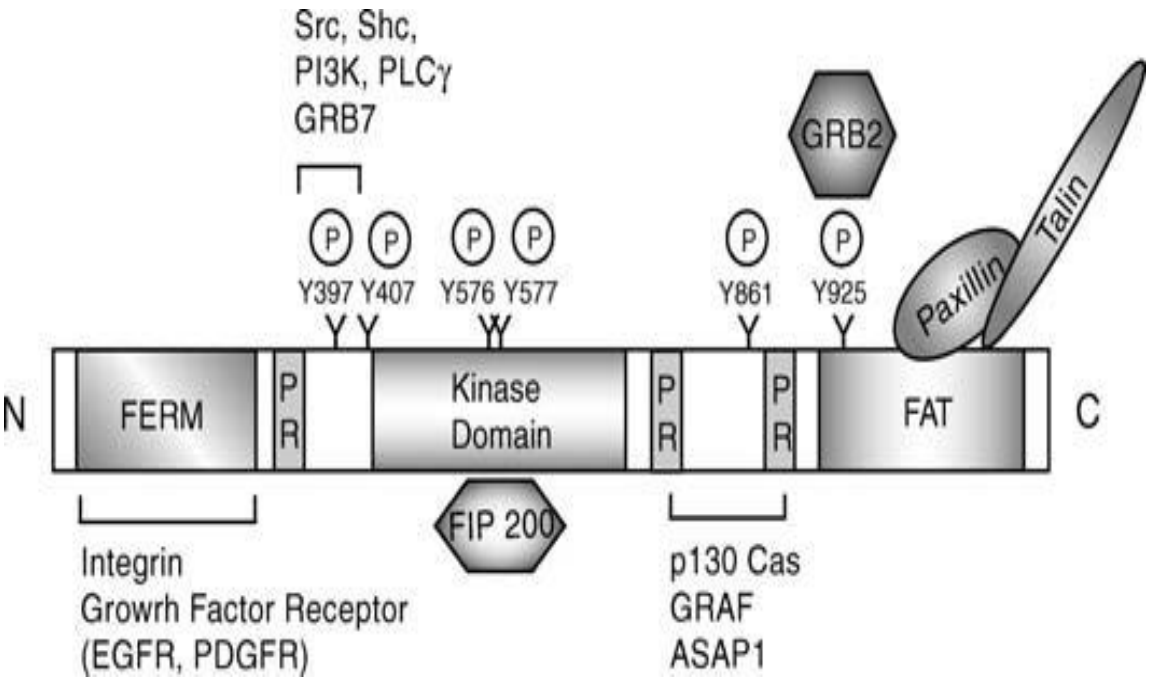

Figure 1.2: FAK linear structure showing major domains and sites of tyrosine phosphorylation (Adapted from Mon *et al.*, 2006).

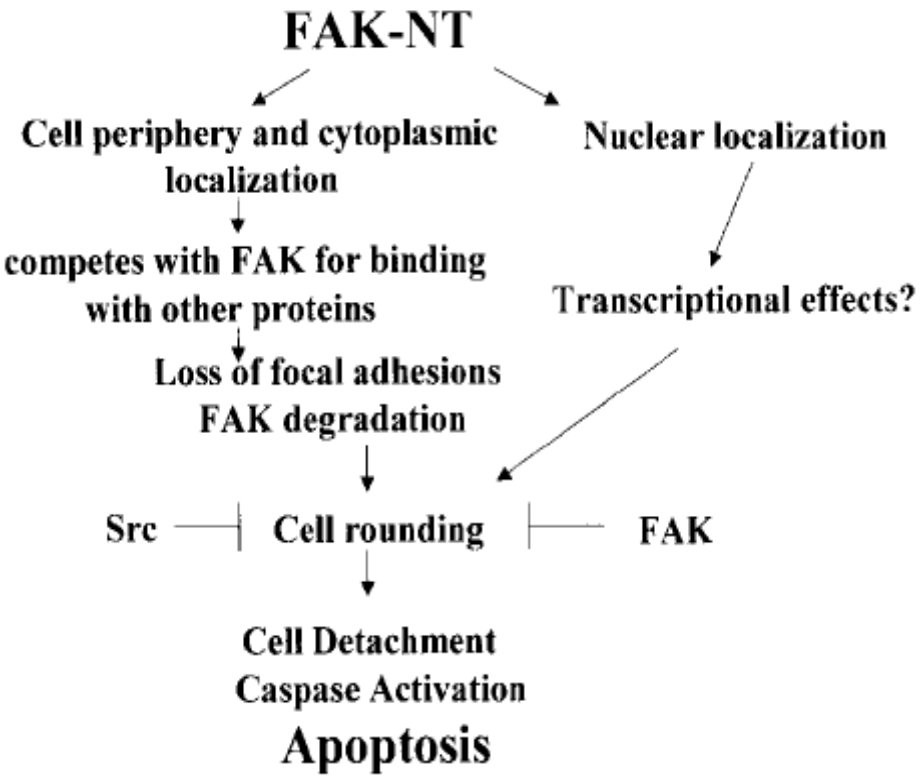

Figure 1.3: FAK-N terminal domain (FAK-NT) induced cell loss of adhesion followed by cell detachment and apoptosis (Adapted from Beviglia *et al.*, 2003).

1.3.3 FAK Phosphorylation, Activation and Inhibition

FAK tyrosine phosphorylation with subsequent activation leads to stimulations of cell adhesion, spreading, invasion and metastasis of cancer cells. Conversely, disrupting FAK association with ECM signalling molecules dephosphorylates FAK specifically at tyrosine residues, inhibits its activity and downregulates several cellular activities (Schwartz & Assoian, 2001; Hynes, 2002; Sawai *et al.*, 2003).

FAK-integrins association at focal adhesions causes Tyr 397 phosphorylation and recruits Src kinases leading to FAK phosphorylation at other residues. FAK phosphorylation at tyrosine residues is an essential event for FAK activation, cell attachment to the ECM, cell adhesion and cell proliferation. The phosphorylation of FAK tyrosine residues is followed by the phosphorylation of intracellular proteins, creation of binding sites for many signalling proteins (Vuori, 1998) and forming signalling complexes at the focal adhesion regions (Ma *et al.*, 2001). Dephosphorylation of FAK tyrosine residues was reported to terminate FAK activity leading to inhibition of specific cellular activities such as migration, invasion and growth (Tamura *et al.*, 1999; Schaller, 2001). FAK activation does not exclusively happen as a result of integrin clustering to focal adhesion plaques, instead, growth factors, neuropeptides and bioreactive lipids are known of activating FAK in integrin-independent manner (Sieg *et al.*, 1999). FAK activation can also be stimulated by platelet derived growth factors (PDGF), and human growth factor (HGF) (Chen *et al.*, 1998; Cospedal *et al.*, 1999). FAK association with Grb2, PI3-Kinase, and p130C signalling molecules enables it to work as a switchable adaptor

protein, situated at a cross junction, controlling different signalling pathways and regulating different cellular activities (Hauck *et al.,* 2002; Beviglia *et al,*. 2003).

Unlike tyrosine phosphorylation, serine phosphorylation appeared to have a negative regulatory effect on FAK. It was observed that serine phosphorylation is normally accompanied by deactivation and dephosphorylation of FAK tyrosine residues and FAK disassembly from signalling complexes (Yamakita *et al.,* 1999). It was reported that mitotic cells or cells at the interphase stage of cell cycle expressed elevated levels of FAK phosphorylated at serine residues (Yamaguchi *et al.,* 1997; Yamakita *et al.,* 1999). Therefore, serine phosphorylation appeared to be involved in cell cycle progression while it may have a downregulatory function on tyrosine phosphorylation.

Generally, FAK phosphorylation at different tyrosine residues has been associated with the development of various types of tumours (McLean *et al.,* 2005). FAK Tyr 397 which is located upstream of the kinase domain is important for supporting adhesion strength, and recruitment of signalling proteins following integrin binding (Michael *et al.,* 2009). Tyr 397 was correlated with higher potencies of cellular migration, proliferation and apoptosis protection (Mon *et al.,* 2006). FAK Tyr 397 has been considered as the only autophosphorylation site on FAK and its phosphorylation initiated FAK phosphorylation at various residues. It has been thought that the phosphorylation of this site is critically required in integrin-mediated FAK signalling (Siesser & Hanks, 2006). The rest of tyrosine residues are thought to be phosphorylated following Src kinases binding to FAK through Src-SH2 binding

domain (Mon *et al.*, 2006). For these reasons, autophosphorylation of FAK Tyr 397 may be considered as the most important event in FAK activation.

FAK association with Src proteins is required for FAK activation. Src binding is essential for N-terminal translocation from the catalytic domain. As a result of that, successive phosphorylations of Tyr 407, Tyr 576, Tyr 577, Tyr 861, and Tyr 925 happen, leading to maximal FAK activation (Schaller, 2001; Mon *et al*,. 2006). Following FAK-Src binding, several signalling molecules will be recruited and activated. FAK association with Src will trigger signalling pathways output amplification leading to the stimulation of various cellular activities (Siesser & Hanks, 2006; Caron-Lormier & Berry, 2005).

FAK Tyr 407 phosphorylation appeared to have a negative effect on cell adhesion, spreading, migration and proliferation (Jeon *et al.*, 2007). Elevated levels of phospho-FAK Tyr 407 were observed when cells were grown under stress conditions, such as during serum starvation, cell cycle arrest and contact inhibition. FAK Tyr 397 phosphorylation was suppressed as a result of FAK Tyr 407 phosphorylation, thus FAK Tyr 407 may negatively affect FAK kinase activity and reduce total FAK activation (Lim *et al*,. 2007). According to another research findings, concurrent activations of FAK, Tyr 407 and Tyr 397 residues were directly correlated with more differentiated colon tumour with less malignant status (Matkowskyj *et al*,. 2003). On the contrary, endothelial cells expressed elevated levels of phospho-FAK Tyr 407 following exposure to vascular endothelial growth factor. Therefore, FAK Tyr 407 could be involved in the transduction of vascular

endothelial growth factor signalling, as it stimulates recruitment of focal adhesion molecules with the increase of endothelial cells migration (Le Boeuf *et al.,* 2004).

FAK Tyr 861 role in cancer was more prominent in breast cancers than any other cancers, since there have been some studies correlating FAK Tyr 861 with breast cancer development. Previous study examined FAK, HER2 protein, phospho-Src Tyr 215 and phospho-FAK Tyr 861 expressions using frozen tissue sections. Western blots analysis showed co-expressions of phospho-FAK Tyr 861 and HER2 proteins (Schmitz *et al.,* 2005). This indicated the critical role of FAK phosphorylation especially at Tyr 861 in regulating cell signalling of human breast cancer. FAK Tyr 861 activation was implicated in FAK-αvβ5 integrins association, and was thought to work downstream of Src kinases. Moreover, FAK Tyr 861 was implicated in triggering signalling pathways leading to vascular development and angiogenesis (Eliceiri *et al.,* 2002).

FAK Tyr 576 and Tyr 577 are located in the kinase domain. Phosphorylation of these tyrosine residues is highly required to attain optimum FAK kinase activity (Jacamo *et al,*. 2007). FAK Tyr 576 and Tyr 577 phosphorylations are important events for restoring autoinhibited conformational FAK activity resulting in increasing FAK phosphorylation (Cox *et al,*. 2006).

FAK Tyr 925 phosphorylation creates binding site for Grb2 protein through focal adhesion targeting (FAT) sequence of the C-terminal domain, and was thought to have a role in cancer cell proliferation (Zhao & Guan, 2009). Besides that, FAK Tyr 925 was implicated in the induction of Ras signal transduction pathway through

association with Src family kinases. Importantly, activation of Ras pathway was expected to happen independently of integrin clustering (Schlaepfer & Hunter, 1996). Therefore Tyr 925 could be involved in the signal transduction pathway in integrin independent manner.

Tyrosine dephosphorylation is as crucial as tyrosine phosphorylation in modulating events happening at focal adhesions. PTP-PEST is one of the important phosphatases secreted at the focal adhesions. Cells lacking of PTP-PEST exhibited a decrease in focal adhesions with migrational defects (Angers-Loustau *et al.*, 1999), however, elevated levels of PTP-PEST resulted in impaired associations and phosphorylation of many focal contacts signalling proteins (Garton & Tonks, 1999). Therefore, PTP-PEST is secreted in a proper balance, and it acts as a key regulator for focal adhesion molecules phosphorylation (Sastry *et al.*, 2002). FAK inactivation can also happen by a tumour suppressor gene known as PTEN which inhibits FAK phosphorylation leading to FAK deactivation (Lipinski *et al.*, 2003).

1.3.4 FAK Signalling

FAK has been involved in integrating and modulating signals of neuropeptides and oncogenes (Burridge & Chrzanowska-Wodnicka, 1996; Schaller, 1997), and in many cancers FAK downstream signalling was shown to work subsequently to integrins activation. Integrins rearrangements on cell surface alter cancer cells phenotypes with changes in cells signalling accompanied by FAK activation (Giancotti & Ruoslahti, 1999). However, according to Yano *et al*. (2004) FAK signalling could

not be directly involved in altered cellular signalling following integrins rearrangements.

Past studies showed that some cellular inhibitory or regulatory cascades were inhibited when cells transformed to cancer cells. That led to the activation of various cellular functions such as migration, proliferation, growth and survival accompanied the stimulation of multiple signalling pathways. Many cellular activities were shown to be more activated in a variety of cancer cells compared to normal cells (Cox *et al*, 2006; Madan *et al.,* 2006). FAK has been acknowledged for a potential role in cancer, and has been contributed to tumourigenesis through the enhancement of different cellular activities (Mon *et al.,* 2006). Previous reports have shown a direct relationship correlating FAK level of expression and cell proliferation, adhesion, migration and survival (Schlaepfer *et al,*. 1999; Hood & Cheresh, 2002). FAK central function in cancer cells was postulated when cellular viability and adhesion to the ECM were inhibited following FAK displacement from focal adhesions. FAK displacement with subsequent dephosphorylation happened when Focal adhesion kinase C-terminal domain (FAK-CD) was used as a negative regulator of FAK (Madan *et al.*, 2006).

1.3.5 FAK as a Scaffold and Src Stimulator Protein

Recently, FAK has also been considered as a scaffold protein which has the capability of nucleating multivalent scaffold complex at adhesion plaques (Carlucci *et al.*, 2008). FAK has been implicated in regulating multiple signalling pathways through its binding to different signalling proteins like paxillin, p130Cas, Src-family

PTKs, p85 subunit of phosphatidylinositol 3-kinase (P13-kinase), Shc and Grb2 proteins. In most cases, binding happens through SH2 and SH3 homology binding sites (Schlaepfer *et al*,. 1999; Jacamo *et al.,* 2007). FAK stimulation of membrane signalling proteins assembled to focal adhesion plaques mainly happen due to the employment of FAK N-terminal domain. This domain helps in connecting FAK with integrins receptors whereby signals will be transmitted downstream triggering FAK activity. Both FAK and integrins especially β-integrin participate in signalling cascades initiation that transmits signals to downstream targets. FAK-integrins association is required for Focal adhesion complex (FAC) establishment within sites of focal adhesions. FAC supports FAK performance as docking site for binding to several pathways mediator molecules (Ilic *et al.,* 1997; Mitra *et al.,* 2005). Apart from that, recruitment of focal adhesion molecules and growth factor receptors such as epidermal growth factor receptor and platelet-derived growth factor receptor (EGFR and PDGFR respectively) to the site of adhesion further stimulate FAK signalling activity (Cohen & Guan, 2005).

FAK involvement in Src activation is not fully understood. It was shown that Src can be activated by integrins independent of FAK. That is, α4β1 integrins stimulate Src activation, and through this activation motility defects can be restored in FAK-null fibroblasts. Src stimulation leads to the activation of p130Cas-associated motility-promoting signalling pathway. This pathway was also proposed to have a critical function in modulating cellular motility, regardless of Src mode of activation. In other words, it can regulate cellular motility either by α4β1 integrins or by FAK dependent manner (Hanks *et al.,* 2003; Mitra and Schlaepfer, 2006). Figure 1.4 shows reciprocal roles for both Src and FAK in working as upstream regulators of

signalling cascades leading to the establishment of cell motility. FAK as a scaffold protein enables it to regulate a range of intracellular signalling molecules namely such as Grb7, Rac, PI3K and others. Accordingly, FAK association with these proteins modulates its activity to accommodate changes in actin and microtubule filaments polymerization, thus directs membrane protrusion and cell polarity toward forward movements.

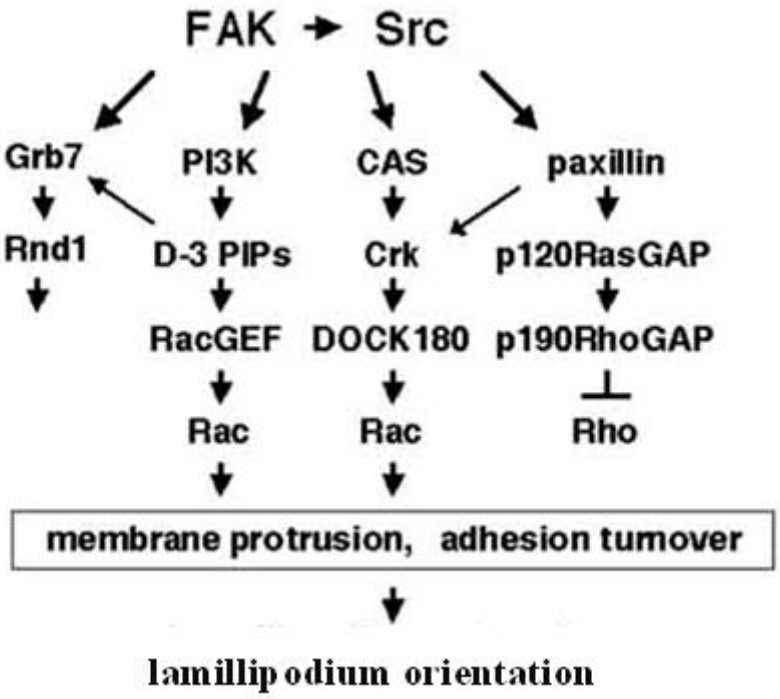

Figure 1.4: Downstream Signalling of FAK/Src is highly required for lamellipodium orientation leading to the promotion of cell motility (Adapted from Hanks *et al.*, 2003).

1.3.6 FAK Signalling and Cell Adhesion

FAK plays a central role in regulating various cellular functions of living cells, and was implicated in cellular force, stress and contraction. Morphological changes happen when cells showed marked decrease in FAK activation. Given that cell spreading is crucial for adherent cell survival, cellular death increased as a result of cellular cytoskeleton defects in FAK deficient cells (Mitra *et al*,. 2005). FAK depletion in endothelial cells produced cells that appeared rounded in shape with less polarity. In addition, FAK deficient cells showed broad lamellipodia with extended protrusions. (Braren *et al*,. 2006). According to previous report, FAK role in adherent cells survival is based on its kinetic role in the formation of adhesive forces. FAK activity was thought to be governed by time-dependent adhesive force mechanosensing responses, where integrin binding and activation are regulated by FAK upregulatory manner (Michael *et al*,. 2009).

FAK was also implicated in protecting ECM-detached cells from undergoing anoikis, and by the virtue of its association with integrin clusters FAK was thought to play a regulatory function in cell adhesion strengthening (Haier & Nicolson, 2000). For instance, FAK contributed to murine FAK-deficient embryonic stem cells survival following detachment from ECM (Ilić *et al.*, 1998).

Fluid flow resistance is a competing shear force against circulating carcinoma cells. FAK was proposed to have an important role in the establishment of early stages of integrin-mediated adhesion of circulating carcinoma cells. FAK is involved in

resisting fluid shear forces, so it exhibits an important function in organ-specific formation of distant metastasis (Von Sengbusch *et al.*, 2005).

1.3.7 FAK Involvement in Cell Migration

Cell migration is a coordinated process resultant from rapid dynamic changes of actin filaments (Mitra *et al.,* 2005). Cell filopodia are formed by parallel bundles of actin, whereas lamellipodia are formed of dense actin meshwork arrangements. Both actin structures are involved in guiding cell motility at cell leading edge, and creating driving forces that enhance forward movement (Etienne-Manneville, 2004).

FAK plays an important role in regulating cell shape and adhesion, besides, FAK's role in migration was proven both *in viv*o and *in vitro* (Hauck *et al.,* 2002; Braren *et al,*. 2006). In previous study, FAK levels increased in migrating cells when wound repair assay had been performed. However, FAK-null mice fibroblast cells failed to migrate but restored migration capacity following FAK transfection (Owen *et al.,* 1999; Lipinski *et al.,* 2003).

1.3.8 FAK Involvement in Cancer Invasion and Metastasis

Several studies have proposed that tumour cells that have the tendency to invade adjacent tissues and metastasize *in vivo* usually showed increased levels of FAK (Gabarra-Niecko *et al.,* 2003). Metastatic tumour cells have the ability to migrate from the place of primary tumour to surrounding or distant tissues. Therefore

migration and invasion are both prerequisites for tumour metastasis (Hsia *et al.*, 2003).

Cancer metastasis requires but not always shared function of matrix metalloproteinases (MMPs) for ECM barriers destruction to allow invasion. MMPs are localized and stimulated at actin-rich cellular points, where association with ECM took place. These contact places which are termed as invadopodia or podosomes are constitutively distinct from focal adhesions (Siesser and Hanks, 2006).

For MMPs-dependent invasion, FAK is involved in MMPs secretion and is known to localize at invasive fronts. Invasion can also happen independent of MMPs secretion but dependent on FAK to regulate or promote different mechanisms of tumour invasions (McLean *et al.,* 2005).

MMP-9 secretion is thought to be stimulated through FAK upstream regulation. MMP-9 secretion requires cell attachment to ECM where variety of signalling proteins including FAK are of importance (Sein *et al,*. 2000). According to previous study (Wu *et al.*, 2005), MMP-2 activation was thought to happen following the stimulation of a cell surface protein known as membrane-type 1 matrix metalloproteinase (MT1-MMP) which is implicated in the cleavage of pro-MMP-2 to its active form. MT1-MMP stimulation was partially thought to be governed by FAK/Src signalling. Increased expressions of MMPs such as MMP-2, MMP-7, MMP-9 and MMP-13 have been implicated in various types of cancer (Sternlicht *et al.*, 1999; Coussens *et al.*, 2002; Egeblad & Werb, 2002).

Figure 1.5 indicates FAK association with cancer development, FAK expression in cancer cells (represented by cells in dark colour) was shown to increase dramatically along with cancer progression. Normal and benign tumours expressed low amounts of FAK, while invasive and metastatic tumours expressed high FAK levels.

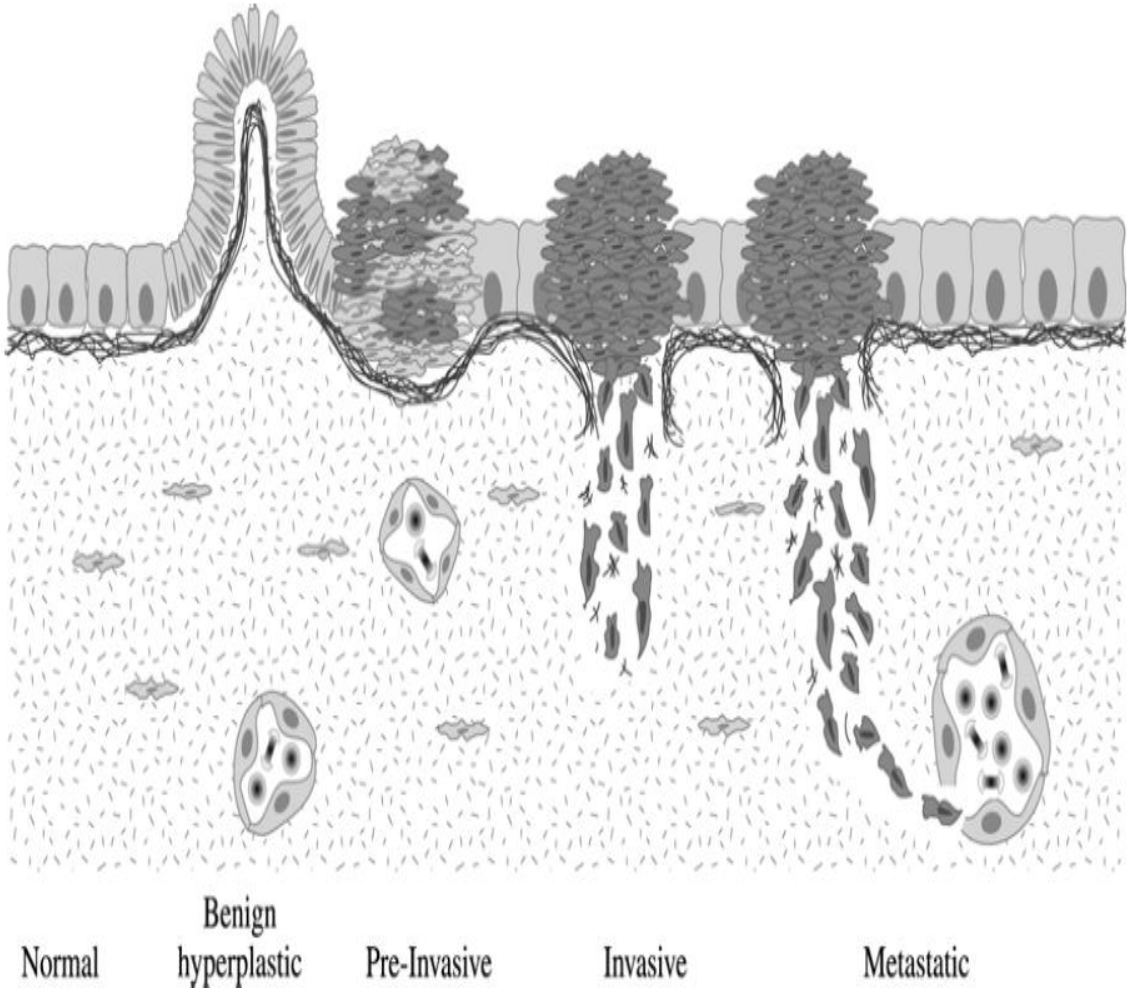

Figure 1.5: Model of FAK association with cancer invasiveness and metastasis. Dark tumour cells represent cells overexpressing FAK (Adapted from Gabarra-Niecko *et al.*, 2003).

1.3.9 FAK Regulates a Dynamic Balance between Cell Survival and Apoptosis

Intracellulalar signalling pathways that regulate and maintain cellular growth and survival are governed by shared signalling networks involving growth factor receptors, integrins and transmembrane receptors (Gilmore, 2005). It is thought that survival or growth signalling pathways interruption can stimulate cell cycle arrest and cell execution. FAK has been associated with cell protection from apoptosis and prolonging survival of anchorage-dependent cells. Reduced FAK levels in some cancer cells resulted in apoptosis (Cance *et al.*, 2000).

Several studies have discussed FAK localization to cell cytoplasm (Sein, 2000; Hauck *et al.,* 2002; Hsia *et al.,* 2003; Mon *et al.,* 2006), though the relation between FAK translocation or localization to cell nucleus and apoptosis is not yet well clarified. In reference to FAK structural functions, FAK N-domain was shown to regulate FAK kinase activity and associate with SUMO protein (Parsons *et al.,* 2003; Cox et al, 2006). SUMO is a FAK modifier which adapts FAK function through its binding to the FAK N-domain, SUMO-FAK association led to FAK localization to nuclei (Golubovskaya *et al.,* 2005; Stewart *et al.,* 2002). However, only recently, the signals that promote or regulate FAK shuttling in and out of the nucleus were limitedly revealed. FAK nuclear localization was also shown to be putatively governed by sequences of leucine-rich nuclear export signals (NES). NES signalling, mainly NES1 and NES2, instigates nuclear protein translocation to cytoplasm (Terry *et al.,* 2007). NES1 sequence is ubiquitously located in the F1 lobe of FAK-N domain, whereas NES2 is situated in the kinase domain of FAK. Both signals are proposed to enable FAK nucleocytoplasmic shuttling (Ossovskaya *et al.,* 2008).

However, since NES2 sequence is not affected by the structural alterations that take place in the FAK kinase domain (Lietha *et al.,* 2007) it was thought to exhibit more important function in the regulation of FAK nucleocytoplasmic shuttling (Ossovskaya *et al.,* 2008).

p53 is a tumour suppressor protein which is considered as one of the most powerful apoptosis triggers upon its activation (Reddig and Juliano, 2005). Usually, when cells are subjected to stabilized growth conditions p53 is maintained at low levels (Vousden, 2002). Cells exposure to stress conditions can enhance p53 stability leading to the stimulation of cell cycle targets like p21Cip/WAF1 (p21). p21 is a cyclin-dependent kinase inhibitor which is stimulated following the exposure to cell death triggers (Oren, 2003; Harris and Levine, 2005). ECM-integrin association was shown to maintain cell survival and stimulate p21 degradation with abrogated p53. The correlation between cell survival and integrin, p21 and p53 is not completely understood (Stromblad *et al.,* 1996; Bao *et al.,* 2002), however could be regulated by FAK nucleocytoplasmic shuttling.

FAK has been implicated in p53 inhibition (Golubovskaya *et al.,* 2005; Cance & Golubovskaya, 2008). Lim et al. (2008) has discussed FAK role in nuclear localization on mouse embryonic fibroblasts cells (MEFs), and suggested that nuclear FAK enhanced cell survival through the degradation of p53 by FAK kinase-independent manner. It was shown that nuclear FAK N-domain works as a scaffold to embrace and stimulate Mdm2 function leading to p53 ubiquitination (Lim *et al.,* 2008). Mdm2 (Murine double minute-2) is one of the p53 regulatory members which regulates p53 level in living cells (Iwakuma and Lozano, 2003).